Postmortem Antigenic Epitope Retention of Galactose-N-Acetylglucosamine in *Bacillus anthracis*-Infected Rhesus Monkey (*Macaca mulatta*) Spleens

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Abstract

BACKGROUND: Anthrax bacilli can be identified by using a monoclonal antibody to galactose-N-acetylglucosamine, a *Bacillus anthracis* cell-wall polysaccharide. We characterized the retention of antigenicity for this polysaccharide in prolonged postmortem autolysis of spleens from anthrax infected monkeys.

EXPERIMENTAL DESIGN: Spleens from infected rhesus monkeys were allowed to autolyze for up to 30 days. To visualize the availability and retention of the polysaccharide at various days of postmortem autolysis, splenic tissue impressions were immunostained by silver enhanced immunogold (IGSS) with a monoclonal antibody against galactose-N-acetylglucosamine.

RESULTS: All tissues had bacilli and fragments of bacilli present from the day of necropsy through 30 days of postmortem observation. Consistent immunocytochemical staining of bacilli occurred from days 3 to 15. In some cases, bacilli stained positively as late as 30 days. Extracellular stain reaction product demonstrating specific immunoreactivity was heavy in day 0 samples and gradually diminished over time to the day 15 samples.

CONCLUSIONS: The antigenic epitope recognized by the monoclonal antibody against galactose-N-acetylglucosamine was preserved with consistency for 15 days postmortem in decaying splenic tissue. Membrane-bound polysaccharide did not appear to be consistently available to antibody for reaction product formation until 3 days postmortem. The polysaccharide also appeared to exist free of the bacillus cell wall, and showed strong immunostaining properties during early postmortem decay.

Additional key words: Monoclonal Antibody, Galactose-N-acetylglucosamine, *Bacillus anthracis*

INTRODUCTION

Postmortem autolysis can hinder the definitive postmortem diagnosis of anthrax in humans, as well as in domestic and wild animals. In a publication describing 42 human cases of inhalation anthrax, anthrax bacilli were recovered by culture in only 20 cases. Twenty cases were culture negative and 2 were not examined. Histology on 15 of the culture-negative cases revealed bacilli (1). Theoretically, the availability of a reliable immunohistologic technique would have facilitated a definitive diagnosis in those 15 cases.

Antibodies have been produced to a variety of *Bacillus anthracis* antigenic subunits. Antibody to a *B. anthracis* cell-wall polysaccharide, galactose-N-acetylglucosamine, has demonstrated a high level of specificity, suggesting it has immunodiagnostic potential (2). It demonstrated superior immunohistochemical signal producing potential in a preliminary screen that also included antibodies to a toxin protein, capsular peptide (poly-D-glutamic acid), and cell-wall protein. However, preservation of the polysaccharide's antigenicity during autolysis has not been reported. In this paper, we report the distribution, availability to antibody, and preservation of the antigenic galactose-N-acetylglucosamine polysaccharide during autolysis of infected tissue.

EXPERIMENTAL DESIGN

Spleen samples from 5 rhesus monkeys infected with the Vollum 1B strain of *B. anthracis,* which had previously served as controls in a drug and vaccine efficacy study (3), were allowed to autolyze for up to 30 days at room temperature (20 to 22° C). Tissue impressions made at days 0, 1, 2, 3, 4, 5, 6, 9, 11, 13, 15, 18, 21, and 30 were Giemsa stained (Baxter, Deerfield, IL) and immunostained by silver enhanced immunogold (IGSS) with a mouse monoclonal antibody against the cell-wall polysaccharide, galactose-N-acetylglucosamine. Pattern and intensity of positive immunostain reaction product were analyzed for all samples.

RESULTS AND DISCUSSION

Two distribution patterns of the IGSS reaction product were observed. The reaction product was either closely bacillus-associated (b/a) or free in the extracellular tissue fluid (e/c) showing no

direct association with bacilli. Mean values for both Giemsa- and IGSS-stained impressions are displayed in Figure 1.

The b/a signal, initially negligible, was strong by 3 days of tissue autolysis. This suggests that the organism-bound polysaccharide was not accessible to antibody binding and/or reaction product formation in fresh tissue, but became increasingly accessible with postmortem autolysis, possibly due to the destruction of the organism's protective poly-D-glutamic acid capsule. By day 3, there were 1 to 20 IGSS-stained organisms per high-power field (org/hpf) on impressions of tissues from all animals. Consistent staining of bacilli was noted from days 3 to 15. Before day 3, IGSS-stained bacilli had an irregular stain pattern (Fig. 2). From day 3 to 9, cell walls of the IGSS-stained bacilli had a uniform stain pattern (Fig. 3), which suggested that antigen was easily accessible to the primary antibody for reaction product formation. Again we noted an irregular cell wall stain pattern in IGSS-stained organisms from tissues subjected to 11 or more days of autolysis. From days 3 to 21, there were equal to or greater numbers of bacilli observed on IGSS-stained impressions than on Giemsa-stained impressions, which suggested that immunostaining with anti-galactose-N-acetylglucosamine enhanced recognition of anthrax bacilli over this period of tissue autolysis. The number of Giemsa-stained microorganisms, < 1 org/hpf on day 18 impressions, gradually rose on days 21 and 30, surpassing the number of IGSS-stained microorganisms on the day 30 impressions. This most likely represents proliferation of contaminating bacterial growth, as significant additional sporulation and new growth of anthrax bacilli would not be anticipated in the airtight microenvironment. In addition to specificity testing described in prior work (2), we tested for and found no cross-reactivity of the primary antibody with *Clostridium sporogenes, B. subtilus, Escherichia coli, Salmonella enteritidis, Pasteurella multocida, Yersinia enterocolitica, Bordetella bronchiseptica, Brucella abortus, Brucella melitensis, and Brucella suis.*

The amount of e/c reaction product, initially heavy, lessened with prolonged tissue autolysis. The heavy concentration on day 0 impressions persisted with no remarkable change until day 3.

From day 3 to 15, reduced yet notable e/c reaction product persisted. With rare exception, there was no remarkable e/c signal from day 18 to 30. This pattern of staining was never observed in stain controls testing for nonspecific binding of the secondary antibody or on impressions prepared from non-infected monkey spleens. This suggests that it was a positive indication of non-cellular bound galactose-N-acetylglucosamine free in *B. anthracis*-infected tissue and fluids.

METHODS

SAMPLE COLLECTION AND SLIDE PREPARATION

Spleen samples for day 0 tissue impressions were taken from 5 untreated *B. anthracis*-infected rhesus monkeys *(Macaca mulatta)* immediately postmortem (3). After gross necropsy observations and splenic sampling, body cavities were closed and the carcasses were allowed to remain at room temperature (20 to 22° C) for 24 hr. Using aseptic techniques, we harvested spleens, diced them into approximate 0.5 cm cubes, and placed them in small capped containers to allow room temperature autolysis for 2, 3, 4, 5, 6, 9, 11, 13, 15, 18, 21, and 30 days. Day 1 tissue impressions made at this time were placed on Superfrost/Plus (Fisher Scientific, St. Louis, MO) microscope slides, air dried, and fixed for 1 hr in 10% buffered neutral formalin. One slide from each group was Giemsa stained to evaluate the relative number of bacilli present.

IMMUNOSTAIN TECHNIQUE

After formalin fixation, the impressions were processed by the following method: a) reverse osmosis (r/o) water, 2 x 5 min; b) Lugol's iodine (4), 1 x 5 min; c) r/o water, 1 x 5 min; d) 5% sodium thiosulfate, 15 sec rinse (4); e) r/o water, 3 x 5 min; f) wash buffer consisting of 0.8% bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, MO), 0.1% fish skin gelatin (FSG) (Sigma Chemical Company), 0.5% triton-X 100 (Sigma Chemical Company), 0.02 M glycine (Sigma Chemical Company), and 2 mM sodium azide (Sigma Chemical Company) in 0.05 M tris-buffered saline (TBS), 2 x 5 min (5); and g) blocking buffer consisting of 0.08% BSA, 0.01% FSG, 5% goat serum (Sigma Chemical Company), and 2 mM sodium azide in 0.05 M TBS, 1 x 15 min. Impressions were drained without rinsing and treated with MAb EAII-6G6 (2)

primary antibody diluted 1:100 in incubating buffer consisting of 0.08% BSA, 0.1% FSG, 1% goat serum, 0.1% triton-X 100, and 2 mM sodium azide in 0.05 M TBS for 90 min; washed with buffer, 2 x 15 min; and then treated with blocking buffer, 1 x 10 min. Impressions were again drained without rinsing and 5 nm gold conjugated goat anti-mouse secondary antibody (Amersham Corporation, Arlington Heights, IL) diluted 1:40 in incubating buffer was applied for 60 min; followed by wash buffer, 3 x 5 min; r/o water, 30 sec rinse; r/o water, 4 x 5 min; and silver enhancement (Amersham Corporation), approximately 10 min.

If the silver autoprecipitation time was < 20 min, the silver enhancement step was split into two 5 min treatments. The impressions were counterstained with working light green solution (6). Nonspecific binding of the secondary antibody was tested for by eliminating the primary antibody application and subsequent steps, proceeding immediately to application of the secondary antibody. Nonspecific binding of the primary antibody was tested for by staining normal monkey spleen impressions at 0, 5, 7, and 9 days of autolysis. A known anthrax positive spleen impression control was also processed with this group. When a day group stained either negative or variable, the group was restained along with a positive control to confirm that the results were not due to a procedural error.

SLIDE INTERPRETATION

Stained impressions were evaluated by brightfield microscopy. The formation of a brown-black reaction product indicated the presence of the target antigen. A subjective score of 0 (negligible) to 4 (heavy) was assigned for each impression, characterizing the amount of e/c reaction product. Scores of 0 to 4 were also assigned for evaluating the number of Giemsa-stained bacilli as well as evaluation of the b/a signal based on 0 < 1 org/hpf, 1 = 1-20 org/hpf, 2 = 21-60 org/hpf, 3 = 61-100 org/hpf, and 4 > 100 org/hpf. The final score assigned for each day of sample evaluation was based on the average for the 5 animals. High-power field (hpf) is defined, in this study, as microscopy with a 40X objective and 10X eyepieces (400X).

TEST FOR BACTERIAL CROSS-REACTIVITY

Using the same immunostain technique described above, we stained formalin-fixed spot slides of *C. sporogenes*, and used formalin-fixed *B. anthracis* (Sterne strain) spot slides as positive controls.

Using the monoclonal antibody labeled with fluorescein isothiocyanate (FITC), we tested for additional cross-reactivity using formalin-fixed spot slides of the following bacteria: *B. subtilus, C. sporogenes, E. coli, S. enteritidis, P. multocida, Y. enterocolitica, B. bronchiseptica, Br. abortus, Br. melitensis*, and *Br. suis*. Impressions of day 3 postmortem *B. anthracis*-infected spleens served as positive controls. Briefly, the impressions were rinsed in distilled water, incubated at room temperature in a humidified chamber for 30 min with normal mouse serum diluted 1:200 in phosphate-buffered saline (PBS), pH 7.4, followed by a 30-min incubation of FITC-labeled monoclonal antibody to galactose-N-acetylglucosamine diluted 1:5 in PBS. The impressions were rinsed in PBS, then in distilled water, mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and examined by fluorescence microscopy.

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FIG. 1. A comparison of mean score values for the relative numbers of Giemsa- and IGSS-stained bacilli (b/a) and amount of extracellular (e/c) IGSS stain reaction product. A subjective score of 0 (negligible) to 4 (heavy) was assigned for each impression. Scores of 0 to 4 were also assigned for evaluating the number of Giemsa-stained bacilli as well as evaluation of the b/a signal based on 0 < 1 org/hpf, 1 = 1-20 org/hpf, 2 = 21-60 org/hpf, 3 = 61-100 org/hpf, and 4 > 100 org/hpf.

FIG. 2. An IGSS-stained chain of bacilli from a spleen with 2 days of postmortem autolysis. The stain pattern for bacilli was irregular and extracellular stain reaction product was moderately heavy (score=3), X1,800.

FIG. 3. An IGSS-stained chain of bacilli from a spleen with 3 days of postmortem autolysis. The stain pattern for bacilli was uniform and the average extracellular stain reaction product was moderate (score=2). In this micrograph, extracellular stain reaction product is mild (score=1), X1,400.

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